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In-Frame TCR δ Gene Rearrangements Play a Critical Role in the αβ/γδ T Cell Lineage Decision

Ferenc Livak,*‡ Howard T. Petrie,† I. Nicholas Crispe,‡ and David G. Schatz*‡
*Howard Hughes Medical Institute
‡Section of Immunobiology
Yale University School of Medicine
New Haven, Connecticut 06520-8011

Summary

Using a quantitative multiprobe Southern blot analysis, we demonstrate the surprising result that a significant proportion of αβ T cells and thymocytes retain T cell receptor δ locus sequences. A substantial portion of the retained δ locus is in a fully V-to-D-to-J rearranged configuration and 20% of these δ rearrangements are functional, significantly less than the 33% predicted for random gene rearrangements. Our observations are in conflict with the idea that αβ and γδ T cells derive from distinct precursors and suggest that commitment of a common precursor to the γδ lineage depends upon expression of a γδ T cell receptor. We propose that the intrathymic T cell lineage decision is determined by a competition between the production of functional γδ and β-pre-T cell receptor complexes.

Introduction

The vertebrate immune system recognizes foreign antigens through the use of antigen-specific receptors encoded by the immunoglobulin and T cell receptor (TCR) gene loci. T cells serve as the major regulators of immune function and can be divided into a predominant αβ and a smaller γδ subset based on their TCR protein expression. Most αβ and γδ T cells develop from a small number of bone marrow–derived precursors in the thymus, progressing through successive steps of maturation characterized by the expression of various surface markers and components of the TCR (for a recent review see Robey and Fowlkes, 1994). Prior to expression, TCR genes are assembled from variable (V), joining (J) and, in some cases, diversity (D) gene segments by a process called V(D)J recombination (for review see Lewis, 1994; Schatz et al., 1992). Assembly of antigen receptor genes is mediated through the coordinate expression of the recombination activating genes *RAG1* and *RAG2* (Oettinger et al., 1990; Schatz et al., 1989) and is absolutely required for mature lymphocyte development (Mombaerts et al., 1992b; Shinkai et al., 1992).

Our understanding of murine intrathymic αβ T cell development has recently been substantially improved through the analysis of various genetically manipulated mouse models. A functional TCR β gene rearrangement promotes cell expansion and developmental progression from the $CD4^-/CD8^-$ double-negative stage to the $CD4^+/CD8^+$ double-positive stage (Mallick et al., 1993; Mombaerts et al., 1992a; Petrie et al., 1990). Functional α gene expression is required for the next stage of development, in which mature single-positive ($CD4^+$ or $CD8^+$) thymocytes are generated from $CD4^+/CD8^+$ cells (Mombaerts et al., 1992a; Philpott et al., 1992). Strict control of TCR β gene rearrangement appears to restrict T cells to the expression of only a single β chain allele (termed allelic exclusion; see Uematsu et al., 1988), while α gene rearrangement is more loosely regulated (Borgulya et al., 1992), allowing individual T cells to express multiple functional α chain products (Hardardottir et al., 1995; Padovan et al., 1993). $CD4^+/CD8^+$ immature thymocytes expressing the αβ TCR are subject to positive and negative selection, which restricts the mature peripheral TCR repertoire primarily to those able to interact appropriately with the host-encoded major histocompatibility antigens (for review see von Boehmer, 1994). It has been shown both in TCR transgenic (Borgulya et al., 1992; Brandle et al., 1992) and in normal mice (Petrie et al., 1993) that surface TCR^+ $CD4^+/CD8^+$ thymocytes continue to express the *RAG* genes, which appear to be down-regulated only after positive selection (Muller et al., 1994; Turka et al., 1991).

Development of the γδ T cell lineage is less well characterized (for review see Raulet et al., 1991), and it remains unclear at what stage the αβ and γδ T cell lineages diverge. Since surface expression of the corresponding TCRs is highly specific for αβ or γδ T cells, it has been speculated that TCR gene rearrangement, expression, or both regulates lineage commitment (see Raulet et al., 1991 and references therein). The TCR δ locus is embedded between the V and J genes of the TCR α locus (Chien et al., 1987a), making the α/δ locus an attractive candidate for regulation of αβ versus γδ lineage divergence. It has been suggested that nonfunctional δRec elements with flanking recombination signal sequences (RSSs) rearrange to 5′ Jα genes and thereby delete the TCR δ locus in thymocytes committed to αβ T cell development (de Villartay et al., 1988; Takeshita et al., 1989).

Studies of excised recombination reciprocal products (Fujimoto and Yamagishi, 1987; Okazaki and Sakano, 1988; Winoto and Baltimore, 1989) and of the transcriptional orientation of the Vα genes (Jouvin-Marche et al., 1990; Wang et al., 1994) strongly suggest that most, if not all, TCR α gene rearrangements occur via a deletional mechanism. Analysis of αβ T cell clones and hybridomas has supported this idea, suggesting that the TCR δ gene was excised and lost upon α gene rearrangement, rendering this locus inaccessible for further studies in αβ T cells (Lindsten et al., 1987). We were therefore surprised to discover with quantitative Southern blot analyses that both total thymus and peripheral T cells (each of which consists

†Current address: Department of Immunology, Memorial Sloan Kettering Cancer Center, Box 341, 1275 York Avenue, New York, New York 10021

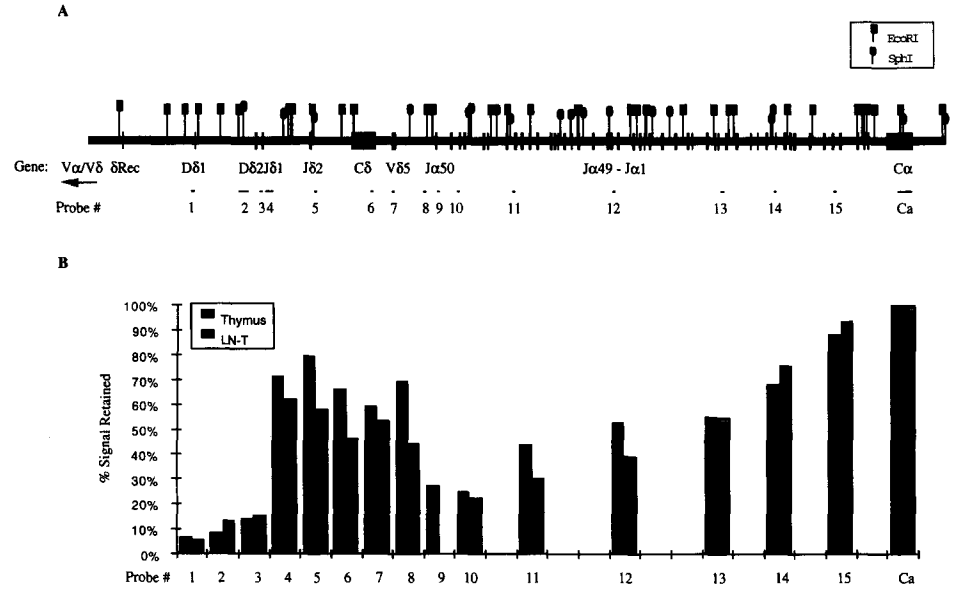

Figure 1. Structure of the Murine TCR α/δ Locus and Summary of Its Quantitative Southern Blot Analysis

(A) Schematic map of the murine TCR α/δ locus encompassing the region from 5′ of Dδ1 through Cα. The region of 5′ of Dδ1 through Jδ2 is derived from published cosmid maps (Chien et al., 1987b); the region from Jδ2 through Cα is a restriction map based on the published sequence (Koop et al., 1992). EcoRI and SphI restriction sites are shown. SphI sites in the δ locus were mapped by Southern blotting (data not shown). Closed rectangles represent the known genetic elements. Individual exons for Cδ and Cα are not shown. The locations and approximate sizes of the 15 probes and the Ca control probe used in this study are shown under the map.

(B) Summary of quantitative Southern blot analyses of the murine TCR α/δ locus in total adult thymus and in peripheral lymph node T cells (LN-T). The horizontal axis is approximately proportional to the map shown in (A). Numbers indicate the probes as shown in (A). The vertical axis represents the percentage of the total germline signal retained for each probe, calculated as explained in Experimental Procedures. The value for the reference probe Ca is set to 100%, assuming no deletion occurs in this region. Values for probes 1–4 were determined using the enzyme HindII, which places 2–4 on individual restriction fragments not spanning an RSS. Note, that probe 4 hybridizes to multiple bands with restriction enzymes SphI–EcoRI or EcoRI (for details see text) and that hybridization of probe 9 was not measured for peripheral T cells. The results shown are averages derived from a number of independent experiments, with the number of determinations made for each probe as follows (in thymus, and in LN-T): probe 1 (2, 1); probe 2 (1, 1); probe 3 (1, 1); probe 4 (2, 1); probe 5 (11, 5); probe 6 (6, 1); probe 7 (7, 2); probe 8 (12, 3); probe 9 (2, 0); probe 10 (17, 6); probe 11 (12, 3); probe 12 (11, 5); probe 13 (9, 3); probe 14 (4, 2); probe 15 (8, 2); Ca (20,8).

of >95% αβ T cells) from adult mice contain large amounts of TCR δ locus sequences, including DNA between Jδ1 and the most 5′ Jα gene segment, Jα50 (Figure 1B). This was particularly striking because the hybridization signal just downstream of Jα50 was substantially lower. Further, we find that the TCR δ locus sequences retained in mature αβ T cells are frequently in a fully VDJ recombined configuration. Analysis of these rearrangements reveals a significant bias against functional in-frame VDJ δ joints in αβ T cells. These observations allow us to propose an alternative model for the lineage divergence of intrathymic αβ and γδ T cells.

Results

Persistence of TCR δ Sequences in αβ T Cells

Analysis of the TCR α locus in normal polyclonal T cells has traditionally lagged behind other TCR loci because its size and complexity did not permit simple and quantitative Southern blot studies. Recent publication of the sequence of the murine TCR Cδ/Cα region (Koop et al., 1992; Wilson et al., 1992) and the development of phosphorimaging methods allowed us to devise a quantitative multiprobe Southern blot technique to investigate the status and progression of TCR α and δ locus rearrangements in total thymus and peripheral T cell DNA. We generated 15 probes specific to various segments of the Dδ1–Cδ–Jα region and used them in various combinations to explore the rearrangement pattern of the Jα locus (Figure 1A, probes 1–15) along with two control DNA probes hybridizing to nonrearranging Cα and RAG-1 sequences. DNA extracted from total or purified thymic subsets, peripheral T cells, and control nonlymphoid tissues (kidney or liver) from young adult mice was restriction digested, transferred, and hybridized, with quantitative analysis of the hybridization carried out on a phosphorimager (see Experimental Procedures).

According to the currently available data (Malissen et al., 1992, and references therein), αβ T cells typically contain V-to-Jα rearrangements on both chromosomes. Since these rearrangements are thought to occur via deletion of the intervening DNA downstream of the V and upstream of the J gene segments involved (Jouvin-Marche et al., 1990; Wang et al., 1994), we expected to detect progressive loss of the germline hybridization signal the more upstream in the Jα cluster a probe is located (Figure 1A, probes 9–15). Furthermore, because the D–J–Cδ region is embedded within the TCR α locus (Chien et al., 1987a),

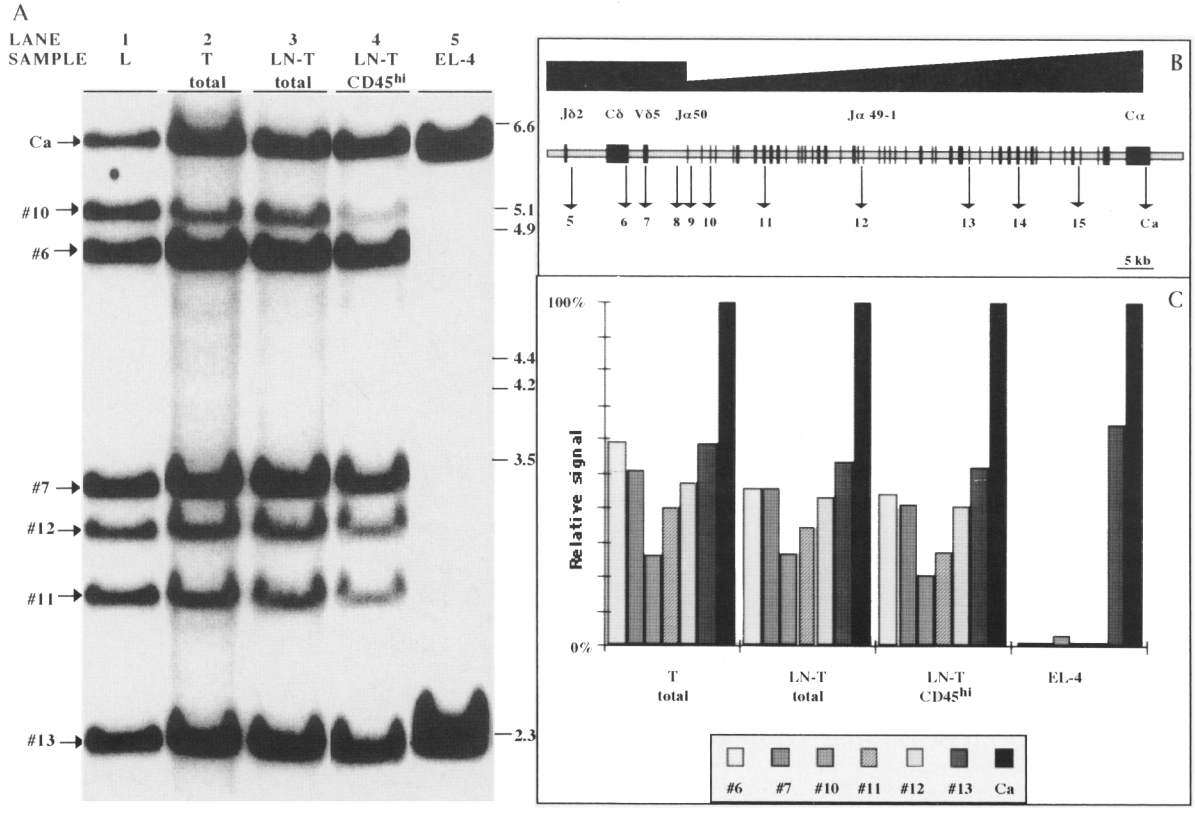

Figure 2. Deletion of TCR α but Persistence of TCR δ Locus Sequences in Total Thymus and Peripheral T Cell Subset DNA

(A) Southern blot analysis of DNA digested with SphI–EcoRI and hybridized simultaneously with probes 6, 7, 10, 11, 12, 13, and Ca. Samples are DNA from liver (L) as a germline control, total adult thymus (T), total peripheral lymph node T cells (LN-T), sorted $CD4^+$ $CD45RA^{hi}$ peripheral lymph node T cells (LN-T $CD45^{hi}$) and EL-4 (an $TCR\alpha\beta^+$ thymoma cell line of C57BL/6 origin).

(B) Portion of the map of the murine TCR α/δ locus relevant to the probes used. Symbols are as in Figure 1A. The schematic representation of changes in hybridization signal intensity throughout the locus is shown above the map.

(C) Bar graph representation of quantitation of the Southern blot shown in (A). The horizontal axis represents the individual DNA samples with data for the probes shown in 5′ to 3′ order. The vertical axis shows the percentage of the total germline signal retained for each probe, calculated as explained in Experimental Procedures.

we anticipated maximum loss of signal with probes 1–8, which hybridize to this region. In contrast, however, we found that the signals obtained with probes 5–8 (corresponding to the J2–Cδ region) were retained at levels of 60%–80% of their total genomic content in total thymus and 40%–60% in peripheral T cell DNA, whereas probes 9 and 10 (corresponding to the most upstream region of the Jα cluster) gave a signal of only 20%–30% of their genomic content in the same samples (see Figure 1B; data for 6, 7, and 10 shown on Figure 2). These results were highly reproducible between experiments and similar results were obtained with several other probes and with another restriction enzyme combination (data not shown). We therefore conclude that both immature and mature αβ T cells (which made up >95% of these preparations) contain DNA corresponding to the TCR δ locus in substantial excess to germline sequences of the upstream portion of the Jα region. Particularly striking is the highly reproducible sharp discontinuity of germline hybridization intensity at Jα50, the most 5′ Jα gene (which is a nonfunctional pseudogene in both mouse and human [Koop and Hood, 1994]; see Figure 1B). This analysis places the 3′ boundary of the hybridization anomaly near the Jα50 gene segment, which in turn marks the border between the TCR α and δ loci. The pattern of hybridization is dramatically different in EL-4, a C57BL/6 origin T cell thymoma, which apparently has both TCR α alleles rearranged and has no TCR δ locus associated sequences retained (Figures 2A and 2C). In a series of Southern hybridization experiments to be reported elsewhere (F. L. et al., unpublished data), we demonstrate that the retained δ locus sequences reside to a significant extent on reciprocal products generated by recombination events involving Jα gene segments.

Complete V-D-J Rearrangements of the TCR δ Gene in αβ T Cells

To map the 5′ end of the persisting TCR δ locus sequences, we analyzed total thymus and peripheral T cell DNA with EcoRI, SphI–EcoRI, or HindII restriction enzyme digestion and hybridized with probes corresponding to the Dδ1–Jδ2 region (1–4; see Figure 1A). We found that sequences between Dδ1, Dδ2, and Jδ1 are extensively de-

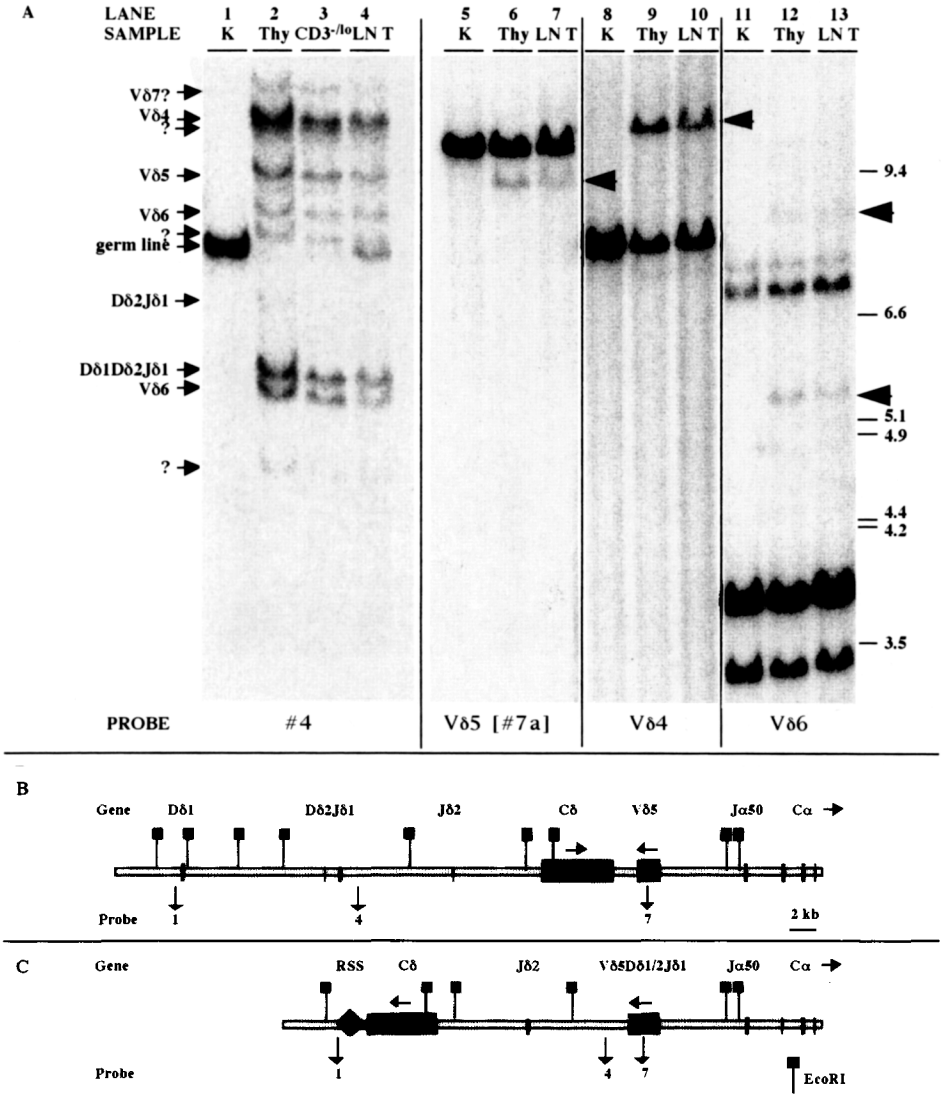

Figure 3. Demonstration of Vδ Rearrangements in Total Thymus and Peripheral T Cell DNA

(A) Duplicate Southern blots of DNA digested with EcoRI, prepared from the same gel, were individually hybridized with probe 4 (lanes 1–4), with probe 7a (which is a probe specific for the Vδ5 gene; lanes 5–7), probe Vδ4 (lanes 8–10), and probe Vδ6 (lane 11–13). For nomenclature, see Elliott et al. (1988). Samples are DNA from kidney (K) as germline control, total adult thymus (T), sorted CD3$^{-/lo}$ thymocytes (only for probe 4), and total peripheral lymph node T cells (LN-T). Bands comigrating on the parallel blots recognized by both probes are considered to represent the specific VDJδ rearrangements (see arrowheads). Other nongermline bands identified with probe 4 are partial rearrangements of D2J1δ and D1D2J1δ (see text) or may represent other VDJδ rearrangements.

(B) Relevant portion of the map of the murine TCR α/δ locus in germline configuration. Symbols are as in Figure 1A. The arrows represent the transcriptional orientations of the Cδ and Vδ5 genes, which are opposite in the germline configuration. Note that probes 4 and 7 hybridize to different EcoRI fragments on the germline chromosome.

(C) Relevant portion of the map of the murine TCR α/δ locus after Vδ5-to-DJδ1 rearrangement. Cδ and Vδ5 are now in the same transcriptional orientation and probes 4 and 7 recognize the same EcoRI fragment of 9.6 kb. The closed diamond represents the reciprocal joint of the RSSs.

leted (see Figure 1B), even if restriction enzymes, such as HindII, were used that would generate small fragments that do not contain an RSS (data not shown). Sequences between Jδ1 and Jδ2, however, are retained to a similar extent (40%–70%) as other more downstream regions (probes 5–8), as judged from probe 4 hybridization to HindII Southern blots (see Figure 1B). Such results would be expected if substantial TCR δ D-to-J1 or V-to-DJ1 rearrangements had occurred in αβ T cells.

This was confirmed to be the case by further hybridization experiments. If EcoRI or the combination of SphI–EcoRI enzymes are used, which place probe 4 on the same restriction fragment as probes 2 and 3, probe 4 shows similar loss of the germline size band. However, in contrast with the other two probes, probe 4 identifies a series of novel relatively intense bands both in total thymus and in peripheral T cell DNA (Figure 3A, lanes 2–4). Hybridization of duplicate Southern blots with probe 7a, which recog-

Table 1. Underrepresentation of Functional Joints in V4D1/2J1δ and V5D1/2J1δ Sequences Obtained from αβ T Cells

	V4D1/2J1δ				V5D1/2J1δ				TOTAL
Tissue	Functional	Nonfunctional	Total	Ratio	Functional	Nonfunctional	Total	Ratio	Vδ4 + Vδ5
Thymus	6	19	25	24.0%	4	20	24	16.7%	20.4%
LN-T	4	18	22	18.2%	6	21	27	22.2%	20.4%
Total Th + LN	10	37	47	21.3%	10	41	51	19.6%	20.4%

Total Vδ4–Jδ1 and Vδ5–Jδ1 rearrangements were PCR amplified from total thymus and peripheral lymph node T cells (LN-T), subcloned, and individually sequenced (for details see Experimental Procedures). Germline sequences were identified by comparison to published genomic (Chien et al., 1987; Wilson et al., 1992), and cDNA sequences (Elliott et al., 1988). The 98 unique sequences consisted of 20 in-frame and 74 out-of-frame junctions, and 4 junctions with in-frame stop codons. Thus, 20/98 = 20.4% of the sequences could encode a complete TCR δ polypeptide (designated as functional), significantly less than the approximately 1:3 ratio expected from random rearrangements. The sequences showed extensive modifications (including deletions and N and P nucleotide additions) at the V–D–D–J boundaries characteristic of adult TCR gene rearrangements.

nizes the Vδ5 gene, demonstrates that one of these novel bands corresponds to V5DJδ rearrangement (Figure 3A, lanes 6–7). The size of this band (9.6 kb) is that predicted for a V5DJδ rearrangement from the genomic sequence (Koop et al., 1992) and is that found in a V5DJδ-containing hybridoma (Takagaki et al., 1989). Other probe 4-positive nongermline bands can be assigned to Vδ4 (Figure 3, lanes 9–10), Vδ6 (lanes 12–13), and possibly Vδ7 (data not shown) rearrangements in total thymus and peripheral T cell DNA. The size of the novel Vδ4 band (13.0 kb) is that found in a V4DJδ-containing hybridoma (Takagaki et al., 1989). Partial D2J1δ and D1D2J1δ rearrangements were identified as bands of the predicted size (Figure 3A) and the identity of the D1D2J1δ band was confirmed by rehybridization to probe 1 (data not shown). Rehybridization of these blots with a control germline probe (RAG-1, data not shown) and subsequent quantitation indicates that Vδ4, Vδ5, and Vδ6 signals correspond to approximately 13%, 8%, and 6% of total genomic DNA content, respectively, which in turn represent approximately 20%, 12%, and 9% of the total retained thymic TCR δ sequences, respectively (see Experimental Procedures for details of calculations). Quantitation of the incomplete rearrangements detected with probe 4 (Figure 3A) indicates that D1D2J1δ and D2J1δ rearrangements represent approximately 15% and 1% of the retained thymic TCR δ sequences, respectively. No more than 2% of the germline sequences are detected in total thymus DNA with probe 4 (the slightly more intense signal in peripheral T cells is due to contamination from nonlymphoid cells). From these data, we conclude that αβ T cells contain 40% of the retained δ sequences in a VDJ rearranged configuration, which is likely a substantial underestimate, since multiple additional Vδ gene segments are known to exist (Wang et al., 1994, and references therein). Similar results for the retention and rearrangement of the TCR δ locus in αβ T cells have been found in an independent study (P. Nakajima, J. Menetski, D. Roth, M. Gellert and M. Bosma, personal communication).

Most TCR δ Rearrangements Are Nonfunctional in αβ T Cells

The VDJδ rearrangements detected in total thymus or peripheral T cells (Figure 3) together represent a minimum of 20%–25% of the total germline hybridization intensity for probe 4, with Vδ4 and Vδ5 rearrangements present at levels of 12% and 7%, respectively. γδ T cells represent a very small fraction of these cell populations (less than 1%; see Experimental Procedures) and, among these cells, only a small fraction should contain rearrangements of any given Vδ gene segment. Therefore, the vast majority (greater than 90%–95%) of VDJδ rearrangements in these samples are derived from αβ lineage T cells. Thus, we could use polymerase chain reaction (PCR) to determine the structure of these rearrangements in αβ T cells without undue concern for contamination from γδ T cells.

To this end, we PCR amplified, subcloned, and sequenced Vδ4–DJδ1 and Vδ5–DJδ1 rearrangements from both total thymus and peripheral T cells. Vδ4 and Vδ5 rearrangements were chosen because we have positively identified them on Southern blots (Figure 3) and because they represent Vδ genes typically used in adult intrathymic γδ T cells (Takagaki et al., 1989). Overall, we found 20 in-frame potentially functional VDJ joints in the 98 sequences determined (20.4%), with a relatively even distribution with respect to source of tissue (thymus or lymph node) or genes (Vδ4 or Vδ5) involved (Table 1). This observed frequency is significantly different ($p < 0.04$) from the approximately 1:3 ratio expected for random rearrangements (see Experimental Procedures) and strongly argues for a selective process based on functional TCR δ gene expression in precursors that can subsequently give rise to αβ T cells (see below). In addition, these data strongly support our contention that the δ gene rearrangements amplified by PCR were derived primarily from αβ T cells, as contamination from γδ T cells would be expected to increase rather than decrease this ratio.

Discussion

We have analyzed rearrangements of the TCR α/δ locus using a reproducible quantitative Southern blot assay and demonstrated not only the presence but also the amount of various V(D)J recombination products during T cell development. In total thymus and peripheral T cells, which represent >95% αβ lineage T cells, germline hybridization signals from the Jδ1–Cδ region are approximately 40%–70% of total genomic content, while germline hybridization

signals from the flanking regions, both upstream of Jδ1 and downstream of Jα50, are substantially lower (Figure 1). This Jδ1–Cδ "hybridization anomaly" is surprising in light of previous hybridoma and T cell line studies (Lindsten et al., 1987) and the relative frequencies obtained from various thymic circle libraries (Okazaki and Sakano, 1988; Toda et al., 1988; Winoto and Baltimore, 1989). Sequence analysis of the retained VDJ δ structures demonstrates that in-frame δ rearrangements are underrepresented, with important implications for our understanding of the mechanisms underlying T cell lineage divergence.

The δ Hybridization Anomaly and the Structure of Retained Recombination Reciprocal Products in α/β T Cells

All available evidence (Jouvin-Marche et al., 1990; Wang et al., 1994) indicates that α rearrangement occurs by deletion, resulting in excision of the δ locus on extrachromosomal circles (Okazaki and Sakano, 1988; Toda et al., 1988; Winoto and Baltimore, 1989). A substantial portion of the excess retained δ locus sequences we have detected lie on broken signal-end molecules in immature thymocytes but not in mature T cells, and a significant portion of the retained TCR δ locus sequences are present on reciprocal signal joint products throughout T cell development and in mature T cells (F. L. et al., unpublished data). The persistence of the extrachromosomal sequences in mature peripheral T cells is compatible with the observation that the majority of $CD4^+/CD8^+$ double-positive and mature $CD4^+$ or $CD8^+$ single-positive thymocytes are not actively dividing (Egerton et al., 1990; Lucas et al., 1993).

Two distinct patterns of TCR α gene rearrangement could explain the sharp discontinuity in germline hybridization intensity seen at Jα50. One possibility is that most rearrangements involving Jα50 are primary events; that is, they are the first α rearrangement to occur on the allele. This is consistent with the hypothesis that TCR α gene rearrangements follow an ordered progression from 3′ to 5′ in the Vα cluster and 5′ to 3′ in the Jα cluster (Thompson et al., 1990), and it is consistent with the hypothesis that the primary event that results in δ locus deletion is δRec–Jα50 rearrangement (de Villartay et al., 1988). However, probe 8 hybridizations of restriction fragments from thymus DNA spanning the Jα50 RSS repeatedly failed to detect a limited number of major Jα50-associated reciprocal products (data not shown; similar results have been reported by Winoto and Baltimore, 1989). This suggests that the rearrangements to Jα50 are quite diverse and is inconsistent with dominant rearrangement of one or only a few δRec elements to Jα50 (de Villartay et al., 1988). Our data are incompatible with the δRec model for a second reason as well: the known δRec elements lie between most of the Vα/δ genes and Jα50 (Wang et al., 1994); thus, primary rearrangement of δRec to Jα50 would preclude most V-to-Dδ rearrangements (including those involving Vδ4 and Vδ6), inconsistent with the frequent VDJδ rearrangements we find. Taken together, our data argue strongly that δRec elements do not play a significant role in murine T cell development.

A second more plausible possibility is that Jα50 is just one of many Jα gene segments involved in primary V-to-Jα rearrangements and, thus, the highly diverse pattern of rearrangements seen at Jα50 is due to secondary α rearrangements occurring on extrachromosomal circles. We favor the idea that most of the reciprocal products containing the Cδ locus we detect are the result of successive rearrangements on persisting recombination byproducts. It is conceivable that recombination reciprocal products containing regions downstream of Jα50 also persist in developing T cells but are not detected in our Southern blot assay because extensive secondary rearrangements cause them to lie on nongermline-sized fragments with the restriction enzymes used. The reason for preferential detection of the Cδ locus would therefore be that this is the only region within the Cδ/α complex that is largely free of recombination signal sequences.

The Relative Order of TCR α and δ Gene Rearrangements in αβ T Cells

The finding that the TCR δ locus is extensively rearranged in αβ T cells raises the question of the relative order of α and δ rearrangements in αβ precursors. If VDJδ rearrangement occurs first, then V-to-Jα rearrangement would delete the rearranged δ locus. Alternatively, if V-to-Jα rearrangement occurs first, then δ rearrangement would take place on the recombination reciprocal products (primarily extrachromosomal circles). Our Southern blot data are consistent with either possibility, but recent elegant results from Lauzurica and Krangel (1994a) argue for the former model. They created mice transgenic for a TCR δ recombination substrate and showed that full assembly of the substrate depended on the presence of an enhancer element. Significantly, while the TCR α core enhancer mediated rearrangement late in thymocyte development (in $CD4^+/CD8^+$ thymocytes) and only in αβ precursors, the TCR δ enhancer mediated essentially complete rearrangement of the transgene much earlier in development (in $CD4^-/CD8^-$ thymocytes) and was active in both γδ and αβ precursors (Lauzurica and Krangel, 1994b). This demonstrates that the δ enhancer has both the appropriate developmental timing and lineage nonspecificity to mediate δ rearrangements well before α rearrangements in αβ precursors.

A second argument in favor of most δ rearrangements occurring before α rearrangements comes from our observation that in-frame δ rearrangements are underrepresented in thymic and peripheral αβ T cells. If δ rearrangements were to occur after α, selection against in-frame δ rearrangements would have to occur in $CD4^+/CD8^+$ thymocytes, since α rearrangement initiates predominantly in these cells. But our Northern blotting data (data not shown) and data from others (Chien et al., 1987a; Held et al., 1990) demonstrate that TCR δ sequences are very poorly transcribed (if at all) in $CD4^+/CD8^+$ thymocytes, making selection against in-frame rearrangements difficult or impossible. Prior rearrangement of δ in the $CD4^-/CD8^-$ precursors of αβ cells would explain the previously puzzling results from the MacDonald group that Cδ sequences are expressed in a substantial proportion of

$CD4^-/CD8^-$ cells and in immature single-positive cells, which are in transition from the $CD4^-/CD8^-$ to $CD4^+/CD8^+$ stages of development (Held et al., 1990). The available data argue that most TCR δ rearrangements precede TCR α rearrangements in αβ T cell precursors.

Our results conclusively resolve a long-standing dispute concerning the structure of the TCR δ locus in αβ T cells. Studies of extrachromosomal circle libraries generated from total thymus DNA resulted in conflicting data detecting either no (Winoto and Baltimore, 1989) or some TCR δ gene rearrangements (Okazaki and Sakano, 1988; Takeshita et al., 1989). Since analysis of extrachromosomal circle libraries rarely yields quantitatively representative results, our genomic Southern blot experiments address this question in a more decisive manner. Our data demonstrate that D–Jδ and complete V–D–Jδ1 rearrangements occur in a large fraction of αβ T cells. We do not have a good explanation for the sharp discrepancy with the results of Winoto and Baltimore (1989). We have repeated our Southern blot analysis on the same murine genetic background they used and obtained essentially identical results to those presented here for δ locus retention and rearrangement (data not shown). In addition, results similar to ours, concerning retention and rearrangement of the TCR δ locus in αβ thymocytes, have recently been derived by others (P. Nakajima, J. Menetski, D. Roth, M. Gellert and M. Bosma, personal communication). Our data are further supported by the finding of overlapping α, β, γ, and δ rearrangements in individual T cell hybridomas (Thompson et al., 1991).

A Model for T Cell Lineage Commitment

The discovery of the extensive persistence of TCR δ gene sequences in αβ T cells has allowed us to analyze the status of this locus directly and quantitatively in αβ T cells. We have clear evidence that the vast majority (>95%) of retained TCR δ loci in αβ T cells contain D-to-Jδ1 rearrangements, and many or most of those contain V-to-DJδ1 rearrangements (Figure 3A). We identified the use of Vδ4, Vδ5, and Vδ6 in these VDJδ structures, which together account for approximately 40% of the retained TCR δ sequences. Sequencing the VDJδ1 rearrangements revealed that approximately one-fifth (20 of 98) of the VDJδ joints are functional, a significantly different result from the 1:3 ratio expected for random rearrangements of Vδ genes in αβ T cells.

There are two main mechanisms to explain the underrepresentation of in-frame VDJδ rearrangements in αβ T cells. Cells with functional δ rearrangements might preferentially develop into γδ T cells or, alternatively, functional δ rearrangements might interfere with the progression/expansion of cells already committed to the αβ lineage. The latter possibility is unlikely because of the substantial amount (≈20%) of functional δ rearrangements found in αβ T cells. In contrast, we show below that the former hypothesis can be incorporated into a model that accommodates this significantly reduced ratio of in-frame joints. Therefore, we favor the idea that a functional δ rearrangement (in conjunction with an in-frame γ allele; see below) promotes γδ T cell development.

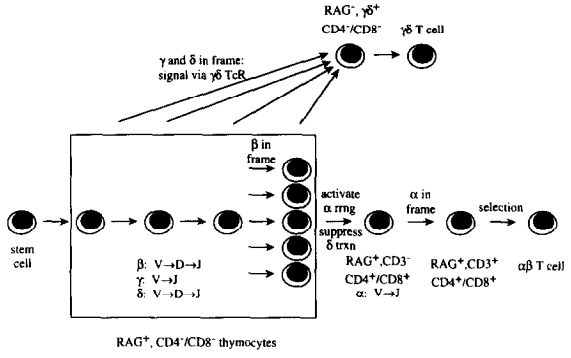

Figure 4. The Competitive Model for T Cell Lineage Commitment

Soon after a stem cell commits to T cell development, it enters the $CD4^-/CD8^-$ compartment (represented by the rectangle) and begins rearranging its β, γ, and δ TCR genes. Whether the cell will become a γδ or αβ T cell is determined by a competition between rearrangement of γ and δ genes on the one hand and activation of α rearrangement and suppression of δ transcription on the other hand. If the cell produces in-frame γ and δ rearrangements and expresses a γδ TCR first, it receives signals through this receptor that result in down-regulation of RAG expression and commitment to the γδ lineage. This can occur at any stage of $CD4^-/CD8^-$ thymocyte development and also in cells with in-frame β rearrangements making the transition from the $CD4^-/CD8^-$ to the $CD4^+/CD8^+$ stages of development (immature single-positive cells). An in-frame β rearrangement leads to cell proliferation, to the $CD4^-/CD8^-$ to $CD4^+/CD8^+$ transition, and to the events that define αβ lineage commitment. See the text for more details.

We propose a competitive model for the divergence of the adult intrathymic αβ and γδ T cell lineages (Figure 4), the important features of which are the following: TCR β, γ, and δ genes are actively rearranged in the same uncommitted precursors ($CD4^-/CD8^-$ thymocytes); cells that produce in-frame functional γ and δ rearrangements and receive appropriate signals through the γδ TCR terminate expression of RAG-1 and RAG-2 (Tatsumi et al., 1993) and commit to the γδ lineage; and in-frame β rearrangement and subsequent signaling through the β–pTα surface receptor (Saint-Ruf et al., 1994) triggers cellular proliferation and promotes further developmental events that lead to commitment to the αβ lineage. Therefore, the lineage commitment decision results from a competition between signaling through the expressed TCR γδ receptor, and αβ lineage commitment events (e.g., activation of α rearrangement and suppression of δ transcription), which occur after signaling through the β–pTα complex.

The competitive model makes the prediction that the percentage of functional δ rearrangements in αβ T cells will be 20%, if one assumes that TCR δ gene rearrangement is completed before lineage commitment occurs, that precursor thymocytes attempt an average of two γ rearrangements, and that the δ locus is subject to allelic exclusion (see Experimental Procedures for details of calculation). We found 20 functional VDJδ1 rearrangements out of 98 sequences (20.4%), in good agreement with this prediction. However, a variety of evidence suggests that at least the first two assumptions used to make the prediction are simplistic. We have shown that approximately 15% of the retained TCR δ loci are in partial D1D2J1 re-

arranged configuration (Figure 3). In addition, some V-to-DJδ rearrangements may occur after the lineage decision is made. Furthermore, the murine genome contains three functional γ loci (Garman et al., 1986), and at least two of them, Cγ1 (Pardoll et al., 1987; Lew et al., 1986) and Cγ4 (Cron et al., 1988; O'Brien et al., 1992), are utilized in adult γδ T cells. It is not known how frequently intrathymic γδ T cell precursors rearrange all four of these γ alleles, but if three γ rearrangements are attempted on average in each cell, then the predicted frequency of functional δ alleles decreases to approximately 15% and, if all four alleles are rearranged, the predicted frequency is roughly 11% (see Experimental Procedures). We emphasize, however, that our 20.4% figure is a maximum estimate of the total proportion of functional versus nonfunctional δ rearrangements in the common T cell precursors, for two reasons. First, incomplete D1D2J1δ rearrangements contribute only to nonfunctional TCR δ alleles and are not sampled by our PCR amplification procedure. And second, rearrangements that occur after the αβ/γδ lineage decision (therefore not present in the bipotential precursors) would be predicted to occur at the random 1:3 ratio. Thus, our results are in good qualitative agreement with the competitive model.

The competitive model also predicts that in-frame Cγ1 and Cγ4 rearrangements should be underrepresented in αβ T cells, which has been shown to be the case for Cγ1 (Raulet et al., 1991). It is interesting to note that rearrangements involving Cγ2, an isotype not reported to be expressed in γδ T cells, show approximately the random distribution of 1:3 in-frame rearrangements in the few instances in which it has been examined (Raulet et al., 1991).

The competitive model has a number of elements in common with the sequential rearrangement model (Allison and Lanier, 1987; Pardoll et al., 1987), but differs from it in maintaining that thymocytes remain uncommitted and bipotential during, and to some extent after the completion of, TCR β gene rearrangement. This is supported by the finding of significant Cδ gene expression in $CD4^-/CD8^-$ and immature single-positive thymocytes but not in $CD4^+/CD8^+$ cells (Held et al., 1990), by the demonstration that late-stage $CD4^-/CD8^-$ thymocytes can develop into both αβ and γδ T cells when injected into congenic recipients (Petrie et al., 1992), and by the recent finding of complete and predominantly in-frame V–D–J β rearrangements in γδ T cells from TCR α knockout mice (Dudley et al., 1994) and in lymph node γδ T cells from normal mice (E. Dudley and A. Hayday, personal communication). Therefore, expression and signaling by an endogenous TCR β polypeptide is compatible with subsequent γδ T cell development, and the outcome of β rearrangement is not an absolute determinant of lineage commitment. The appearance of normal numbers of γδ T cells in TCR β knockout mice (Mombaerts et al., 1992a) also makes it clear that β rearrangement and expression are not required for γδ T cell development. It therefore seems likely, as depicted in Figure 4, that commitment to the γδ lineage can occur at any stage of $CD4^-/CD8^-$ thymocyte development after the onset of γ and δ rearrangement.

Our data are inconsistent with a number of the current models for T cell lineage commitment. The different lineages model (Dent et al., 1990, and references therein), which proposes that the αβ and γδ lineages are independent and that lineage commitment is not affected by the outcome of gene rearrangement events, is incompatible with our finding that functional δ rearrangements are underrepresented in αβ T cells. And our data conclusively rule out a more specific version of this model, according to which lineage divergence is accomplished through the mutually exclusive rearrangement of the TCR α and δ loci (Winoto, 1991; Winoto and Baltimore, 1989). Lineage divergence has also been ascribed to the lineage specific action of a γ transcriptional silencer, postulated to block expression of functional γδ receptors in αβ lineage cells even if the corresponding γ genes are in frame (Haas and Tonegawa, 1992; Ishida et al., 1990). The simplest version of this model implies that one-third of full VDJδ rearrangements should be in frame in αβ T cells, which is in conflict with our data.

TCR γδ or β transgenic mice should provide useful experimental models to study the αβ versus γδ T cell lineage commitment process, but have thus far yielded conflicting results. The competitive model predicts that early expression of the transgene(s) should interfere with the development of the opposite lineage. TCR β transgenic mice, as predicted by the model, lack intrathymic γδ T cells (von Boehmer et al., 1988). TCR γδ transgenic mice, however, show blockade of αβ T cell development in some cases (Bonneville et al., 1989; Dent et al., 1990) but not in others (Dent et al., 1990; Ishida et al., 1990). The competitive model suggests that the exact timing and perhaps level of expression, as well as the specificity and signaling ability of the transgenic γδ receptor, should be critical in determining the effect on αβ T cell development. Therefore, the contradictory results obtained may be the result of small variations in these parameters between different transgenic mice.

The competitive model implies that precursors of γδ and αβ T cells are identical and that their developmental separation is achieved through the differential response mediated by the γδ versus β–pTα receptor complexes. Future studies are required to determine the identity of the ligands interacting with these receptors, the nature of the signals that result from these interactions, and the specific molecular events that complete the lineage commitment process.

Experimental Procedures

Cell Preparations

Female 4- to 8-week-old C57BL/6 and 5-week-old AKR/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Single cell suspensions from thymus and all major lymph nodes were prepared in a tissue grinder. Thymocyte subsets were electronically sorted after staining with anti-mouse TCR αβ antibody (Clone H57-597) conjugated with streptavidin phycoerythrin (Pharmingen, San Diego, California). Purity of TCR $\alpha\beta^-$/TCR $\alpha\beta^{lo}$ and TCR $\alpha\beta^{hi}$ thymocytes was determined by reanalysis with independent staining reactions (see below) and found to be greater than 98% and 95%, respectively. Total peripheral T cells were prepared by panning on anti-murine immunoglobulin (Sigma)- coated plates (Wysocki and Sato, 1978). Purified cells were analysed on a FACScan flowcytometer by staining with one or more of the following monoclonal antibodies: anti-mouse CD3 (clone 29B),

Table 2. Sequence of PCR Primers Used to Generate Probes 5–15, Vδ4, and Bδ6

Probe	5′ Primer sequence	3′ Primer sequence	Size
5	GGCGGATCCTACAGCTCAGAAGGCCTTCAG	GGCGGATCCATGTAGTTGTCCTATGG	390
6	GGCGGATCCCAGGCTTCCAACTTCTCAG	GGCGGATCCTCGCCTCAGGAGAGG	420
7	GGCGGATCCAGATGCCTGACAACCTGG	GGCGGATCCCTCTGAGCTGGTCAGTG	400
8	GGCGGATCCATTCCCCATCATCCCCG	GGCGGATCCTTTGCATTATCTCTGCCTG	400
9	GGCGGATCCTGAAAATTGCTGGAGGGAG	GGCGGATCCTTCCTTGGGTACCAGGAG	400
10	GGCGGATCCTGGGGGAAAGCCACTGGTGC	GGCGGATCCCAGGGAAAGGACGAAGGTC	425
11	GGCGGATCCTGCTGGGAAGAGAG	GGCGGATCCTTGGTGGAGACCCTAGG	420
12	GGCGGATCCTTTCCCGTTCACAATG	GGCGGATCCTTGGATGGGGAAG	425
13	GGCGGATCCTGGGCTCTGGCTAGG	GCGGGATCCAGTGGGCATGGAGCTGG	500
14	GGCGGATCCTTCCGCCCTCCATTTTCCG	GGCGGATCCCTCATTCAAACTCATGG	450
15	CGGGGATCCTACAGCGAGCGAGTAG	GGCGGATCCAGTCACTGGCAGGTCAG	430
Vδ4	AGCGGATCCGGCCGTTCTTCCTGT	CGGAATTCCTGCCCTCCTTGCTGTTT	480
Vδ6	AGCGGATCCTTGGCTTTCTCTGGAT	GCGAATTCTGTGAGGCTGAAATGAC	500

All primer sequences are shown from 5′ to 3′. Underlined sequences indicate artificially introduced restriction enzyme sites to facilitate subcloning. The size of the PCR product amplified from C57BL/6 kidney DNA is indicated on the right in base pairs. For information on other probes see Experimental Procedures.

anti-mouse CD4 (clone H129.19), anti-mouse CD8a (clone 53-6.7) all from GIBCO BRL; anti-mouse TCR αβ, anti-mouse CD45RB, anti-mouse B220 (clone RA3-6B2), all from Pharmingen; and anti-mouse TCR γδ (clone GL3) from Boehringer Mannheim. Purity of $CD3^+$ peripheral T cells was greater than 95%, of which 1% was γδ TCR^+. In total thymus preparations less than 0.5% γδ TCR^+ cells were observed.

Nucleic Acid Preparation and Hybridization

High molecular weight DNA was prepared from total homogenized kidney or liver and single cell suspensions of various T cells populations by proteinase K digestion/phenol–chloroform extraction (Ausubel et al., 1989). DNA was restriction digested with the indicated enzymes, electrophoresed through 0.7%–0.8% agarose gel, and transferred to Gene Screen Plus nylon membrane (New England Nuclear) on a Posiblot apparatus (Stratagene). Hybridization was carried out in the presence of 50% formamide (Ausubel et al., 1989) with random hexamer primed [α-^{32}P]dCTP-labeled probes (Feinberg and Vogelstein, 1983). Autoradiography and quantitative analysis were performed on a PhosphorImager with ImageQuant 3.0 software (Molecular Dynamics, Sunnyvale, California). The retained hybridization signal (in percent) shown on Figures 1B and 2B is calculated as follows:

$$\text{Percent of signal retained} = \frac{\text{signal for probe of interest in T cell}}{\text{signal for germline control in T cell}} \times \frac{\text{signal for germline control in kidney}}{\text{signal for probe of interest in kidney}} \times 100$$

The hybridization signals with probe 4 for complete and incomplete TCR δ rearrangements (Figure 3A, lane 2) were measured similarly, and the hybridization intensity relative to the control kidney sample was calculated as above after RAG-1 rehybridization. This yields the percentage of probe 4 germline hybridization intensity contained in each band. To calculate the percentage of retained δ locus sequences contained in each band, we corrected for the fact that only approximately 65% of the probe 4 hybridization intensity is observed in total thymus DNA (Figure 1B). For example, V4DJδ rearrangements, which represent 13% of total probe 4 germline hybridization intensity, were calculated to represent 13/0.65 = 20% of the retained probe 4 sequences in thymus. These data were confirmed by measuring the relative hybridization signals of Vδ5, Vδ4, and Vδ6 rearranged bands individually (Figure 3A, lanes 6, 9, and 12, respectively).

DNA Sequencing

TCR V4(D)Jδ and V5(D)Jδ sequences were PCR amplified, restriction enzyme–digested at artificially introduced enzyme sites (Table 2) and directionally subcloned into pBluescript II KS(+) vector (Stratagene). DNA prepared from individually picked clones was double-strand sequenced on an ABI automated sequencer with the Taq Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, California). Note, that the VDJ joints are inserted in opposite orientation relative to the *LacZ* gene of the vector, therefore no full-length fusion proteins with β-galactosidase can be produced. Multiple stop codons are found in each frame of Vδ4 or Vδ5 sequences in context of *LacZ*, suggesting that selection against any particular frame in bacteria was unlikely.

Probes

The following probes (see Figure 1A) were used: probe 1, identical with the 600 bp HindIII fragment designated probe A (a gift from D. Raulet; Korman et al., 1989); probe 2, 1.2 kb BglII–EcoRI fragment of pTAE7-7 (a gift from A. Winoto; Winoto and Baltimore, 1989); probe 3, 900 bp HindII–PvuII fragment of pTAE7-7; probe 4, 1.0 kb NdeI–XbaI fragment of pTAE7-7; probes 5–15 were PCR amplified with the indicated primers (Table 2). Probe 6 was 1.5 kb EcoRI fragment of cDNA of the Cδ gene (a gift from L. Lefrancois; Elliott et al., 1988), or was PCR amplified with indicated primers. Germline control probes: Ca, 400 bp BamHI–HindIII fragment of exons 2–4 of the Cα gene from cDNA of a D10α clone (gift from S. Hong; Hong et al., 1992) or 1.7 kb XhoI genomic fragment 3′ of the coding sequences of the Cα gene from cosmid CaBS2 (provided by D. Sant'Angelo); RAG-1, 630 bp EcoRI–MluI fragment of RAG-1 cDNA (Schatz et al., 1989). PCR amplifications were carried out in thermocyclers (MJ Research, Watertown, Massachussetts) under standard conditions (Ausubel et al., 1989) on C57BL/6 kidney DNA, followed by subcloning into pBluescript II KS(+) vector. Vδ4- and Vδ6-specific probes were derived by PCR using the indicated primers (Table 2).

Calculations

The percentage of δ rearrangements predicted to be in frame in αβ T cells by the competitive model was calculated as follows. Consider 81 precursor cells, all of which initiate δ rearrangement. Two-thirds (54 cells) will be out of frame on the first attempt ($\delta^{-/0}$), and the remaining 27 will be in frame ($\delta^{+/0}$). Of the 54 $d^{-/0}$ cells, two-thirds (36 cells) will be out of frame on the second allele ($\delta^{-/-}$) and can never become a γδ but only an αβ T cell. Of the remaining cells (27 cells [$\delta^{+/0}$] and 18 [$\delta^{+/-}$]), each will attempt γ rearrangement on one allele and then on another, as seen above for δ. The total rate of generation of γ^+ cells will be 3/9 plus 2/9 = 5/9 in both δ^+ populations, thereby giving rise to 15 plus 10 = 25 γδ T cells. The remaining γ^- cells can become αβ T cells (a total of 12 [$\delta^{+/0}$] plus 8 [$\delta^{+/-}$] = 20). We analyze only αβ T cells and only fully rearranged (i.e., no δ^0) alleles. Therefore, we calculate 36 plus 36 plus 8 = 80 δ^- alleles and 12 plus 8 = 20 δ^+ alleles, resulting in a ratio of 20/100 = 20% in frame.

If a third γ allele is assumed to rearrange in the 20 γ^- cells (12 cells [$\delta^{+/0}$] plus 8 cells [$\delta^{+/-}$]), then the contribution of δ^+ and δ^- alleles by these cells is reduced to two-thirds of its previous value. Combining these with the 72 δ^- alleles from $\delta^{-/-}$ cells (whose fate is unaffected by additional γ rearrangements) yields a result of 14.7% in-frame ratio. Similarly, if a fourth γ allele is then allowed to rearrange, the contribu-

tion of δ alleles by γ^- cells is again reduced to two-thirds of its previous value and the result becomes 10.5%.

The analysis of the statistical significance of the deviation of our result from the random (1/3) expectation was derived from the binomial equation for the probability (P) of obtaining exactly k in-frame rearrangements out of N total sequences: $P(N,k) = C(N,k)(2/3)^{N-k}(1/3)^k$, where $C(N,k) = N!(N-k)!/k!$. Of the 98 sequences, 20 junctions were in frame and functional, while 4 others were in frame but nonfunctional due to insertion of an in-frame stop codon. The calculation of statistical significance was based on the total number of in-frame junctions (24) irrespective to their coding potential. The probability of 24 or fewer in-frame rearrangements occurring by chance out of 98 total sequences was then calculated by summing the results for k = 1 to 24.

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Note Added in Proof

It has recently been demonstrated by others that both TCR δ and γ rearrangements are predominantly (80%) out of frame in αβ lineage T cells, and that TCR β rearrangements are commonly in frame in γδ T cells (Dudley, E. C., Girardi, M., Owen, M. J., and Hayday, A. C. [1995]. αβ T cells and γδ T cells can share a late common precursor. Curr. Biol., in press.